NOTE

Gram-scale production of a basidiomycetous laccase in *Aspergillus niger*

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We report on the expression in *Aspergillus niger* of a laccase gene we used to produce variants in *Saccharomyces cerevisiae*. Grams of recombinant enzyme can be easily obtained. This highlights the potential of combining this generic laccase sequence to the yeast and fungal expression systems for large-scale productions of variants.

[Key words: Multicopper enzyme; Bioreactor; *Aspergillus niger*; *Saccharomyces cerevisiae*; Glycosylation]

Achievements in biocatalyzed reactions require robust methods of production for enzymes coming from natural biodiversity and/or for evolved enzymes. The multicopper oxidase laccase (*p*-diphenol: O₂ oxidoreductase, EC 1.10.3.2) is a popular biocatalyst for potential technological and bioremediation processes in addition to its use in organic synthesis (1). So far, laccases from plant, bacteria, ascomycetous and basidiomycetous fungi have been expressed in bacteria, yeasts, filamentous ascomycetes, basidiomycetes or plant with various success reaching occasionally expression levels close to a gram per liter in complex cellular contexts (2,3). Improvement in the production of single laccase variants in a given host may be obtained through variations of cultivation conditions, induction or mutagenesis (mainly through directed evolution) (4,5). For practical reasons, prior to any rationalization, it may appear crucial first to select a generic laccase encoding sequence that is generously expressed in heterologous expression hosts. Having previously used the yeast *Saccharomyces cerevisiae* to produce LAC3 (LAC3^{Sc}) and several variants with yields in the mg/L range (6–8), we report here on the production of grams of LAC3 (LAC3^{An}) in the industrially relevant generally recognized as safe (GRAS) ascomycete *Aspergillus niger*.

We successfully integrated the *lac3* cDNA to the genome of *A. niger* [strain D15#26 (*pyrg*[−]) (*prtT*) (*phmA*) (9)] and selected the LAC3 highest producing transformants for their ability to oxidize ABTS (volumetric activity). We followed a construction strategy and selection procedures identical to those previously published (10). Prior to any adjustments, the laccase titer obtained in flask culture was multiplied by 10 comparing *S. cerevisiae* to *A. niger* recombinants

(data not shown). In *A. niger* it is known that both the pH regulation and the type of agitation of automated bioreactor systems have a marked influence on heterologous expression. So, scouting for fermentation parameters was conducted on 0.5 L cultures with a BIostat-Q bioreactor. Initial fermentation conditions (20 g/L glucose, pH 5.5, aeration of 0.6 VVM (volume of air/volume of culture medium/min), 2 × 10⁶ spore/mL inoculum) allowed us to reach a maximum of 6000 U/L (~120 mg/L, using syringaldazine as substrate) laccase in 7 days before the enzyme started to degrade. From this initial fermentation we varied individually four cultivation parameters susceptible to influence the laccase production and established step-by-step the conditions for a routine fermentation (Fig. S1). Briefly, varying the glucose concentration from 20 g/L to 50 g/L laccase activity increased from 18,000 U/L to 24,000 U/L; doubling, then tripling the aeration rate laccase activity increased substantially from 18,000 U/L to 42,000 U/L; on the other hand, either lowering the temperature from 28°C to 26°C or reducing the inoculum size by 2 orders of magnitude led to a significant decrease of laccase activity. Adjusting these four parameters resulted in a substantial increase in LAC3 titer to a 42,000 U/L corresponding to 840 mg/L of recombinant enzyme produced in a 0.5 L fermentor. In the subsequent up-scaled fermentation (Fig. 1A), owing to the fact that LAC3 is the major secretion product (Fig. 1B), a simple concentration and one chromatographic purification step (anionic exchanger, 50% final yield) allowed us to recover 2.3 g of LAC3 with a specific activity of 50 U/mg and a satisfactory level of homogeneity as controlled by UV/VIS spectroscopy ($A_{280}/A_{610} = 15$) and denaturing gel electrophoresis (SDS-PAGE, Fig. 2A).

All together, this represents a substantial 200-fold increase in laccase production as compared to our previous heterologous production in *S. cerevisiae* (6,8). The yield reported here is comparable to that reported for an ascomycetous laccase produced in

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TABLE 1. Apparent kinetic parameters for LAC3^{An} and LAC3^{Sc} in acetate buffer (100 mM, pH 5.7) at 30°C.

Apparent kinetic parameters ^a	ABTS ($\epsilon_{420\text{ nm}} = 36000\text{ M}^{-1}\text{ cm}^{-1}$)		Syringaldazine ($\epsilon_{525\text{ nm}} = 65000\text{ M}^{-1}\text{ cm}^{-1}$)		Guaiacol ($\epsilon_{465\text{ nm}} = 12100\text{ M}^{-1}\text{ cm}^{-1}$)	
	LAC3 ^{An}	LAC3 ^{Sc}	LAC3 ^{An}	LAC3 ^{Sc}	LAC3 ^{An}	LAC3 ^{Sc}
k_{cat} (min ⁻¹)	8378 ± 126	13955 ± 300	4756 ± 157	11038 ± 271	3087 ± 79	5278 ± 511
K_M (μM)	734 ± 54	875 ± 87	16.2 ± 1.5	22.1 ± 1.2	4743 ± 375	4457 ± 272
k_{cat}/K_M (min ⁻¹ μM ⁻¹)	11.4	16.0	282.4	499.5	0.65	1.2

^a Apparent K_M and k_{cat} values were obtained from the initial rate (v) enzyme concentration (E) and substrate concentration (S) according to Michaelis–Menten equation [$v = k_{\text{cat}} \times E \times S/(K_M + S)$] (non-linear regression fitting using Prism program, Graphpad, San Diego, CA, USA). Laccase concentration was determined by the Bradford colorimetric assay using a lyophilized sample of pure laccase as the standard (1–50 μg/mL) (15).

Likewise, host specificities in post-translational processes probably account for the differences in catalytic efficiency observed for the enzyme purified from the two hosts (i.e., LAC3^{An} is globally 55–70% less efficient than LAC3^{Sc}, see Table 1).

LAC3^{An} pH tolerance (Fig. S2), copper ions content (4 atoms of copper), spectroscopic signatures and overall structure are features markedly close or even indistinguishable to those of LAC3^{Sc} (Table S1). The general understanding on glycosylation fit a general non-specific effect on protein properties such as stabilization of conformation, protection against proteases and secretion of proteins (16). Accordingly, effect of glycans is likely related to local variations in the structure of the protein rather than to a global change in the enzyme structure (17). Therefore, despite the specificity of their respective glycosylation machineries, the *S. cerevisiae* and the *A. niger* expression systems can be profitably used in combination for protein engineering as shown recently with the expression of an evolved laccase mutant in *A. niger* (18). Moreover, the amount of laccase produced in *A. niger* opens the way for a valorization of laccases properties in the construction of artificial metalloenzymes (7,19,20).

In conclusion, we have described the heterologous expression of a basidiomycetous laccase gene in a GRAS organism which secretes the recombinant enzyme at a level close to the g/L. Simple adjustments of fermentation operations in a bioreactor allow the production of grams of the recombinant laccase. As the recombinant enzyme is the main secretion product of the host its subsequent purification is largely facilitated. This enzyme is representative of the laccase family. The form produced in *A. niger* is functionally similar to the one previously produced in *S. cerevisiae*. Perfectly amenable to synthetic evolution, secreted at substantial levels in industrially relevant organisms, this multicopper oxidase is a potential generic for the development of low cost means of production of tailored biocatalysts.

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.jbiosc.2013.06.013>.

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